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Title: Aqueous Decontamination of Biological Pathogens Using Modified Fenton Chemistry

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Aqueous Decontamination of Biological Pathogens Using Modified Fenton Chemistry

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Abstract

An approach to decontamination of biological pathogens is discussed. Specifically, the performance of an aqueous modified Fenton reagent is examined. A modified Fenton reagent formulation of cupric chloride and ascorbic acid is shown an effective sporicide under aerobic conditions. Results are given for the modified Fenton reagent de-activating spores of *B. globigii*. A biocidal mechanism is proposed that is consistent with our experimental results and

independently derived information found in the literature. This mechanism requires diffusion of relatively benign species into the interior of the spore where dissolved O_2 is then converted through a series of reactions into hydroxyl radicals that perform the killing action.

INTRODUCTION:

Microbial life is abundant, tenacious, and is often very difficult to control. Organisms including viruses, bacteria, and fungi are often characterized by an ability to spread easily, reproduce rapidly, and thrive in conditions that can destroy higher life forms. Many of these organisms are completely harmless to humans and play an important role in many ecosystems and natural processes. Some organisms though cause human diseases and exclusion or destruction of these organisms is important to prevent or block the spread of disease.

In addition to the problem of normal infections, the world is faces with the rapidly growing problem of "super bugs" or bacteria that have developed a resistance to one or more antibiotics or disinfectants. In its annual Report on Infectious Diseases, the World Health Organization (WHO) warned that many major infectious diseases are becoming resistant to the antibiotics uses to treat them (Press Release WHO/41, 12 June 2000). As an example of this problem, WHO noted that in Estonia and Latvia, as well as parts of

Russia and China, over 10% of those afflicted with tuberculosis have strains resistant to both of the most powerful antibiotics used to treat the disease.

Many of these drug resistant diseases are acquired and spread in hospitals and hospitals are being forced to take extraordinary measures to guard against patient infection. Hospitals and medical facilities commonly use sterilizing agents on patient contact surfaces, operating rooms, and medical instruments in order to combat growth and spread of disease. The sterilizing agents commonly used include formaldehyde and glutaraldehyde which is cancer causing and put at risk hospital personnel. These agents as well as oxidants react directly with the outer membrane or coat of bacterial cells and spores. Though effective, these agents are also generally very highly reactive toward organic materials in general and some inorganic materials, thus causing corrosion or erosion. Because of the reactivity of these agents toward many different compounds, they could react with and thus be consumed by things other than their intended targets, thus becoming less efficient due to their tendency to cause collateral damage. Further, these substances often proved toxic as has been mentioned. Use of these agents was thus conducted with a conscious understanding that such use would often eventually degrade the surfaces or materials sanitized and with extreme caution to avoid contact with a patient, health care worker, or the environment. It would thus be a significant

improvement to develop a sterilant that was relatively nonreactive to non-target organic and inorganic materials and that was non-hazardous to humans or to the environment.

H.J.H. Fenton first studied the catalytic decomposition of hydrogen peroxide by transition metal ions [1] in 1894. This work led to over 100 years of studies on the Fenton "reagent" [2,3] in which in its simplest form it was shown that the hydroxyl radical (HO*) was the species responsible for the reagents high oxidation efficiency. The basic mechanism

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + HOO^* + H^+ \qquad (1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^* + HO^- + HOO^* \qquad (2)$$

shows that the transition metal, in this case iron, is cycled between an upper and lower oxidation state by it's interaction with hydrogen peroxide and that the net reaction

$$H_2O_2 \longrightarrow HO^* + H_2O \qquad (3)$$

employs the transition metal effectively as a catalytic agent forming hydroxyl radicals. Subsequent reactions of hydroxyl radicals [2] with organic molecules include 1) hydrogen abstraction, 2) addition reactions to double bonds, and 3) oxidation reactions.

Fenton reactions can be operative in biological systems especially where hydrogen peroxide is formed during the course of normal cell function. Low levels of transition metals such as iron and copper are normally present in biological systems where they aid

in respiration and biological polymer conformation. For example, during cell respiration, the bio-molecule NADPH is oxidized by dissolved oxygen to form the superoxide molecule O_2^-.

$$NADPH + 2O_2 \longrightarrow NADP^+ + H^+ + 2O_2^- \quad (4)$$

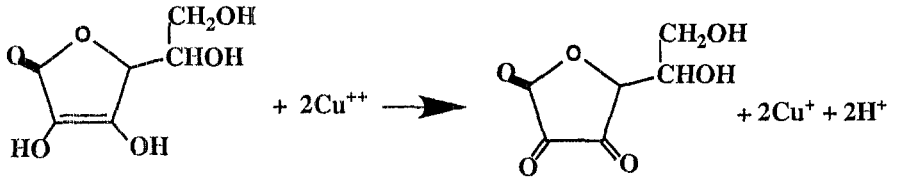
The superoxide is converted to hydrogen peroxide in a slightly acid medium by superoxidedismutase (SOD). SOD

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (5)$$

If transition metals ions are present, the Fenton reaction can form hydroxyl radicals that in many cases can damage the biological organism. In fact, there are numerous studies [4] indicating the toxic nature of iron and copper and their role in aging of biological systems.

Cells efficiently scavenge hydrogen peroxide and free radicals through the glutathione peroxidase/reductase and catalase [5] systems. In addition to these natural defense mechanisms, drugs such as erythromycin [6] and vitamins such as ascorbic acid [7] are employed as added defense mechanisms against free radical formation. Ascorbic acid (Vitamin C) has long been recognized as an efficient free radical scavenger [8] but under certain conditions of high concentration and the presence of transition metals and dissolved oxygen, it can be a pro-oxidant [9,10)]. Copper ions are especially active [11] in forming hydroxyl radicals under aerobic conditions in the presence of ascorbic acid

[12]. In biological systems, copper (II) complexes with DNA or RNA upon which a site-specific formation of hydroxyl radicals occur that causes DNA/RNA strand scission [11]. At high concentrations of ascorbic acid (AA), copper (II) is rapidly reduced to copper (I) with the formation of the oxidized form of ascorbic acid, dehydroascorbic acid (DAA).



(6)

Ascorbic acid

AA

Dehydroascorbic acid

DAA

Under aerobic conditions however, copper (I) is spontaneously oxidized back to Cu (II) with the subsequent formation of hydrogen peroxide through the superoxide intermediate [13]

$$2Cu^{+} + 2O_2\,(aq) \longrightarrow 2Cu^{++} + 2O_2^{-} \qquad (7)$$

$$2O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2 \qquad (8)$$

with the net reaction being: $AA + O_2 \xrightarrow{Cu^{++}} H_2O_2 + DAA.$ (9)

Thus under conditions of excess ascorbic acid, where Cu (II) is continually kept reduced by ascorbic acid to Cu (I), dissolved oxygen is converted to hydrogen peroxide. The peroxide is subsequently converted to hydroxyl radicals by the Fenton reaction:

$$Cu\ (I) + H_2O_2 \longrightarrow HO^* + HO^- + Cu\ (II) \qquad (10)$$

Both copper and iron combined with hydrogen peroxide have been investigated [14, 15] as a substitute for conventional disinfectants [16-22]. Although conventional disinfectants suffer from poor efficacy and staff sensitization, 2% glutaraldehyde is a chemical of choice for disinfection of medical devices and for use in hospitals. Fenton based disinfection using hydrogen peroxide can be more efficient with essentially no side effects compared to glutaraldehyde [14]. The investigations reported here extends the concept of Fenton based disinfection and shows that hydrogen peroxide can be replaced with aqueous dissolved oxygen and ascorbic acid to effect a much higher kill effectiveness than either conventional or hydrogen peroxide-metal based disinfectants with no adverse side effects.

MATERIALS AND METHODS:

The results presented here are limited to surrogate biological pathogens. Surrogates have been chosen to closely replicate specific properties of biological pathogens of interest. For example, we have examined the following organisms:

Spore Forming Bacterium: *Bacillus globigii*

Vegetative Bacterium: *Erwinia herbicola*

Virus: *Bacteriophage MS-2*

Bacillus globigii is a durable non-infectious gram positive spore forming bacteria common in certain soils and is a fair surrogate to *B. anthracis*. *Erwinia herbicola* is the standard simulant for the causative agent of bubonic plague and other non-sporulating, gram-negative bacteria. This surrogate is a vegetative, non-spore forming, gram-negative bacterium that is non-infectious to animals and man, but is found on plant leaves. *Bacteriophage MS-2* is an RNA virus, which infects *E. coli* that is relatively easy to handle and is a good simulant for a viral disease agent. The complete nucleotide sequence is known for this organism. All of these surrogates are easily grown in culture, easily detected, and require only Biosafety Level 1 handling precautions.

Spores are generally much harder to deactivate than vegetative bacteria and viruses, and thus generally represent a stringent test of a decontamination reagent. Therefore, we have concentrated this effort on the effectiveness of Fenton reagent against spores of *Bacillus globigii*.

The *Bacillus globigii* used in these studies was *var. Niger* grown from stock obtained from Dugway Proving Grounds using the following protocol. A "2 x SG" - Schaeffer's Sporulation Media (stock solution #1) was prepared from 16 g/liter of Difco nutrient broth, 0.5 g/l of $MgSO_4 \bullet 7H_2O$, and 2.0 g/l of KCl. Additional stock solutions included

10% glucose, 1 M Ca $(NO_3)_2$ 0.1 M $MnSO_4$, and 0.001 M $FeSO_4$. The components of stock solution #1 were dissolved one at a time, in the order indicated, in one liter of triply distilled H_2O. 100-ml aliquots were poured into media bottles. The caps were loosened and autoclaved in a pan of water for 20 minutes on slow exhaust. The bottles were allowed to cool completely, the caps were tightened and solutions were stored in the dark until use. As prepared, the Schaeffer's Nutrient Base lasts approximately one week. The complete sporulation media, "2 x SG", was made by adding quantities of the other stock solutions to the Schaeffer's Nutrient Base after the base was autoclaved. The quantities added were: 10ml/l of glucose solution, 1 ml/l of calcium nitrate, 1 ml/l of manganese sulfate, and 1 ml/l of iron sulfate. All stock solutions were filter sterilized using a 0.22 μm Acrodisc filter.

The fermentation process used to produce spores consisted of the following. First, 2.8 liters of Schaeffer's media were added to a five-liter fermentor with one 200-ml spore suspension ($1x10^2$ spores/ml). Five days were allowed for growth and sporulation (30°C, moderate aeration) to occur. After harvesting the culture was heat shocked at 65°C for 15 minutes to kill any vegetative cells. The spores were then harvested by centrifugation (10,000 rpm for 25 minutes) and washed with triply distilled sterile water, 10 times. The spores were then re-suspended in 500 ml of sterile triple distilled water. To enumerate the number of viable spores in the

resulting preparation, a dilution series was performed on the suspension followed by plating on nutrient agar plates to determine population of spores. The average of at least two plates was taken.

Specifically, the procedure for small cultures and fermentation inoculate consisted of the following steps:

1. Streak a freezer stock for isolation on NA and incubate overnight in 30°C incubator.
2. Take 1-2 colonies from the plate and inoculate two 5-ml aliquots of Schaeffer's Nutrient Base. Incubate overnight in 30°C shaker.
3. Inoculate 190 ml of sporulation media, "2 x SG", with the two 5 ml cultures. Do this in a 1 liter Erlenmeyer flask to ensure aerobic environment. Incubate in 30°C shaker. Allow to become turbid (24 h) and use directly as an inoculum for a 3-L fermentation or to continue incubation for sporulation.
4. Check for sporulation by making a wet mount and examining using phase contrast microscopy.
5. Once there is > 80 % spores, heat shock the culture for 15 minutes at 65°C to lyse remaining vegetative cells. Harvest by centrifugation (10,000 rpm for 25 minutes). Wash spores with triply distilled sterile water, 10 times.
6. Re-suspend pellet in 40 ml of triply distilled sterile water.

7. Perform a dilution series on culture using NA plates to determine population of spores. Take the average of at least two plates.

Slightly different procedures were used for surface decontamination studies versus liquid decontamination studies. Test coupons were prepared on glass surfaces as follows:

1. Sterile 2 cm^2 glass coupons (scored and cut from 1 x 3 in. frosted glass microscope slides) were prepared.
2. The coupons were aligned with the smooth size exposed. The glass coupons were then inoculated with *B. globigii* stock solution, approximately 5 x 10^7 spores/ml. The pipette was fitted with a new tip for each coupon.
3. The inoculated coupon(s)were dried at room temperature overnight in a dessicator containing Drierite.
4. The coupons were aseptically transferred (spore end down) into sterile 13x100 mm glass test tubes just prior to pickup for testing. Controls (which would not undergo exposure to the gas) were also prepared.
5. Following testing, the coupons were placed in a known amount of neutralization agent (sodium thiosulfate) and then sonicated for 120 minutes to remove spores.

Separates tests proved the sonication procedure capable of near 100% recovery off the coupons and that the sonication did not induce deactivation of the spores.

6. The spore population was then determined by performing triplicate serial dilutions of the spore suspension. Serial dilutions were prepared for each sample, using sterile phosphate buffer as the diluent. Serial dilutions (generally over 5-7 logs of dilution) on both samples and controls were performed. Plating was accomplished using spread plates, drop plates, or pour plates. Both NA and BHI was used as media. We found that when BHI is employed as the growth medium, 48 h are typically required to accurately distinguish colonies with the pour plate method. For this reason, plates were incubated for 24-hr minimum, 48 h maximum at 30°C.
7. The D values and magnitude of kill were then determined by counting colonies.

Figure 1 shows a typical SEM image of dried spores on a glass coupon following desiccation. We generally inoculated the smooth side of the glass coupons with the spore suspension because spores placed on the impervious smooth surfaces are the most difficult to deactivate.

For tests in liquid solution spore suspensions were prepared as previously described and the following steps were executed:

1. A 1 ml aliquot of spore culture was placed in test tubes.
2. Tubes were spun for 10 minutes at 16,000 x g to pellet the cells. The supernatant was discarded.
3. 3 tubes were re-suspended in the reagent to be tested (the precise volume and tube size varied from one test to another).
4. A fourth sample tube was re-suspended in sterile triple distilled water as a control.
5. The tubes were allowed to sit for the specified exposure time at room temperature..
6. Dilution, temperature, and/or addition of neutralization agents (sodium thiosulfate) quenched the reaction.
7. The sample tubes and the control were the spun at 16,000 x g for 10 minutes to pelletize the cells. The supernatant was removed.
8. The spores were rinsed two times in an appropriate diluent.
9. The spores were then re-suspended in 1 ml of fresh sterile phosphate buffer.
10. Serial dilutions were then prepared for each tube, using sterile phosphate buffer as the diluent. Serial dilutions (generally over 5-7 logs of dilution) on both the test samples and the controls were performed. Plating was accomplished using spread plates, drop plates, or pour plates. Both NA and BHI were used as media.
11. The D values and magnitude of kill were then determined by counting colonies.

In all cases, a standard serial dilution of the stock spore culture was carried out along with every set of decontamination tests to verify that the culture was still viable and that the number of colony forming units (CFU/ml) was constant in time. In addition, the phosphate buffer solution was tested for spore contamination by plating the buffer with no dilution. All procedures were carried out using standard sterile or aseptic bacteriological techniques in sterile, laminar flow biohazard.

Reagent grade $CuCl_2$ and ascorbic acid (AA) were employed in the Fenton reagent. Copper chloride and AA were dissolved in deionized, tap water, or salt-water (2M). A 1-% surfactant (3M FC-170 fluorinated surfactant) was also added in some experiments to reduce surface tension of the aqueous solution. Exposure time of spores to the reagent was usually 30 minutes and was performed at ambient temperature (25° C). Scanning electron micrographs (SEM) were performed using a JOEL 6300 instrument on spores that were mounted on an aluminum sample holder by drying a water suspension of spores using an ambient temperature dessicator. The mounted spores were gold coated to reduce charging effects.

RESULTS:

Figure 2 shows the results of 30-minute exposure of *Bg* spores to various formulations of Fenton reagent. Note that copper ion (0.6 M) **with** hydrogen peroxide

(B) shows very poor kill effectiveness with and without added AA. This is very surprising since other experiments with *Erwinia herbicola* shows excellent kill with this formulation. Fenton formulations **without** hydrogen peroxide show excellent kill (~-4-5 orders-of-magnitude) with both an ionic strength and surface tension dependence. The ionic strength dependence can be seen in the results obtained with and without NaCl (see C, E, and F in Figure 2). The effects of a change in surface tension achieved by the use of a surfactant can be seen in Figure 2-G. The highest kill effectiveness (% surviving=$5X10^{-6}$) was obtained with 0.06M $CuCl_2$, 0.1M AA, 2M NaCl, and 1% by weight surfactant. Other experiments performed with AA, 2M NaCl, and surfactant alone (no copper) showed no kill what so ever.

Figure 3 shows the effect of removing dissolved oxygen from all the aqueous solutions. Nitrogen was bubbled (1 hour) through the deionized water to make anaerobic Fenton reagents as well as the anaerobic Bg spore suspension solution. The extent of oxygen removal from the interior of the spores is unknown but the removal of oxygen in this case made a large difference (2-log) in spore kill indicating that the presence of oxygen is important in the kill mechanism. This can be seen in C and D of Figure 3.

Figure 4 shows the effect of aerobic modified Fenton reagent aging. Over time ascorbic acid decomposes into diketo-L-gulonic acid, oxalic acid, and L-threonic acid [23] and loses its reducing powers. In fact on day one, the $CuCl_2$ solution, which is blue-

green to begin with, converts to a colorless solution when ascorbic acid is added indicating the reduction of $Cu^{++}(CuCl_2)$ to $Cu^{+}(CuCl)$. On day 14 the blue-green color had returned to the solution but was removed when additional ascorbic acid was added. The copper remains active as long as it is in its reduced state therefore a disinfectant solution can be rejuvenated by the addition of ascorbic acid.

In order to gain additional information on the kill mechanism, experiments were performed with $Cu^{+}(CuCl)$ versus $Cu^{++}(CuCl_2)$ with and without ascorbic acid. Figure 5 shows the results of employing three variants of the formulation: Cu^{+} (0.06M) with salt (2M), no ascorbic acid, and 1% surfactant, which is labeled as B;-the same formulation as B but with ascorbic acid, the results of which are labeled as C; and finally the same formulation as C but with $Cu^{++}(CuCl_2)$ instead of Cu. Results using the last formulation are labeled as D in Figure 5. As employed, Cu^{+} exhibits much less kill (4 log) than Cu^{++} under the same conditions probably because DAA is not formed but is the form of AA needed to enter the spore (see discussion).

SEM analysis was employed to image the results of spore exposure to the modified Fenton reagent. Figure 6 shows the SEM results after 30-minute (A) and 16 hour (B) exposures of *Bg* spores to modified Fenton reagent. Note first that the spore coat appears in tack even after 16 hours of exposure, that there is essentially no difference in appearance between 30 minute and 16 hour exposure, and that the spore coat appears to

be unaffected compared to live spores (Fig. 1) except for a few spores being broken open. These results are in contrast to the effect that other free radical producers, such as ozonated water, have on *Bg* spores [24], as shown in Figure 7. Figures 7 and 8 show that ozone attacks and dissolves the spore coat and an over long exposure time eliminates any trace of the spore. Clearly the kill mechanisms for ozone and modified Fenton reagent are very different and that ozone kills by attacking the coat while the modified Fenton attacks the inner workings of the spore, leaving the coat relatively untouched.

DISCUSSION:

Knowledge of the disinfection mechanism of pathogens is important for optimizing kill efficiency and minimizing undesired effects on surroundings and high level multicellular organisms. Disinfectants can be classified into two groups according to whether the kill mechanism originates from inside the pathogen or outside. For example, ionizing radiation can form free radicals inside a cell (or spore) that then attack the interior life giving machinery. Alternatively, chemical sterilants can be employed that attack the cell or spore coat from the outside ether causing lysis or blocking nutrient or oxygen uptake. Exterior disinfectants are commonly used in hospitals [15-22] and water treatment systems (ozone, chlorine compounds). These exterior disinfectants are usually

powerful oxidizers that indiscriminately attack both inanimate organic matter and living organisms and are generally toxic to higher level organisms [22-glutaraldehyde] or form toxic byproducts. Ionizing radiation kills by forming hydroxyl free radicals (from the decomposition of water) that then attack DNA or enzymes within the cell. By controlling the point of irradiation in an organism, discrimination can be achieved and serious side effects controlled. The detailed disinfection mechanism for bacterial cells and viruses by metal ions [25] is well understood but the mechanism operating in spore sterilization [26] is not as well developed. However, the environments within the spore along with spore characteristics that provide long-term survival strategies have been well-elucidated [27]. A spore kill mechanism of the sterilant under investigation is proposed using information on metal ion attack on cells.

The sterilant studied in this investigation, a modified Fenton reagent, appears to demonstrate an interior kill mechanism that does not require addition of a strong oxidizer such as hydrogen peroxide (see Fig. 3, B and C). After sterilization, the body of the spore is left relatively intact with no obvious degradation of the spore coat (compare Fig. 6 and Fig. 1). This is in contrast to ozone, which clearly attacks the spore coat (Fig. 7) and of course can attack all organic matter. The detailed mechanism by which the modified Fenton reagent operates is unclear but a mechanism can be proposed that is at least consistent with the data presented here and data available in the literature. Figure 3

shows that hydrogen peroxide generated hydroxyl radicals produce only 1-log of kill. In the absence of hydrogen peroxide but in the presence of ascorbic acid, dissolved oxygen results in a 5-log kill. The elimination of dissolved oxygen from the aqueous solutions reduces the kill by ~2-log (Fig 3, C to D). It is unclear how much dissolved oxygen was actually removed from the spore interior by bubbling nitrogen through the spore suspension so the results shown in Figure 3(D) may represent only a partially anaerobic situation with respect to dissolved oxygen contained within the spore. **It appears that hydroxyl radicals produced from the conventional Fenton reaction between copper ions and hydrogen peroxide (equations 1, 2, and 3 where iron is replaced by copper) are not effective in killing when produced externally to the spore.** A comparison of experimental results C, D, and E in Figure 2 indicates that ionic strength is important. The addition of a salt whose anion readily passes through the cell wall may result in more cations (i.e. copper) being driven into the spore in order to satisfy the electroneutrality and osmotic constraints. A comparison of E and G in Figure 2 indicates that reduction of liquid surface tension also plays an important part in the mechanism; possibly providing more intimate contact of the reagent with either individual spores or clumps of spores. In addition, the results shown in Figure 5 indicate that when equation 6 is turned off, i.e., by using Cu^{+}, ascorbic acid is not oxidized to its dehydro-form (DAA), and the kill is reduced by ~4-log. Aerobic water was used so in the case of B and C (Fig. 5), some of

the Cu^{+} may have been quickly oxidized to Cu^{++}. In case C, the Cu^{++} could then oxidize AA to DAA thus increasing the kill. Evidently, the formation of DAA is important in the kill mechanism. The SEM micrographs in Figure 6 indicated that spore coats are intact and the data in Figure 2 show excellent kill. Thus, it appears that the kill mechanism must be an interior one with reactants diffusing through the spore coat and forming free radicals inside the spore. Relatively little is known about diffusion of water and small molecular weight species into the spore interior [27]. Figure 9, obtained through the application of *in situ* atomic force microscopy technique [28], though shows the effect upon a dried spore when immersed in water. Extensive uptake of water is observed and it is assumed that dissolved oxygen in the water would also be brought into the spore interior. Many studies [29, 30, 31, 32, 33] of ascorbic acid entry into **cells** indicate that Vitamin-C is transported in its DAA form and subsequently reduced to AA. Since DAA is structurally similar to glucose, it has been proposed that glucose transporters [29, 31] mediate its mechanism of entry into cells. In some cases, the transport is found to be Na^{+} ion dependent [33, 34] which may also explain the impact NaCl has on biocidal activity. All though these studies indicate a possible path for AA entry, there have been no definitive studies of the transport mechanism of AA entry into **spores**. Never the less, our data is consistent with the idea that DAA is the species being transferred into the spore. Once inside the spore, DAA needs to be reduced to AA in order to produce

sufficient Cu^{+} concentration from Cu^{++}. Then dissolved O_2 can be converted to hydroxyl radicals. None of our data sheds light on which copper ion (Cu^{++} or Cu^{+}) is transported into the spore, but reaction 6, converting Cu^{++} to Cu^{+} and DAA, is very fast (milliseconds) compared to typical diffusion rates, so it is assumed that Cu^{+} is exterior to the spore and is the ion transported into the spore. After formation of hydroxyl radicals interior to the spore, these highly reactive species attack either the DNA in a site-specific manner [10] or attack enzymes necessary for spore conversion to a bacterial cell. Investigations that are more extensive than these will be needed to determine precisely the mechanism of spore kill under these conditions

CONCLUSION:

Decontamination of a biological pathogen simulant *B. globigii* was studied. Specifically, the use of an aqueous modified Fenton reagent was examined. A reagent formulation of cupric chloride and ascorbic acid is shown an effective sporicide under aerobic conditions. Results were obtained for a variety of formulations. It was found that sodium chloride and a surfactant enhanced the biocidal potency. The additional ions may promote mass transfer through the spore coat while the surfactant may enhance reagent contact with individual spores or spore clumps. A mechanism was proposed that is consistent with our experimental results and information found in the literature. This

mechanism requires diffusion of relatively benign species through the spore coat. Once interior to the spore, dissolved O_2 can be converted into hydroxyl radicals. The free radicals then perform the killing action.

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28. Atomic Force Micrographs of *B. globigii* taken with a Dimension 3000 (Digital Instruments) in the tapping mode, by Dr J. J. Weimer, Laboratory of Materials and Surface Science, The University of Alabama in Huntsville.

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Figure Captions

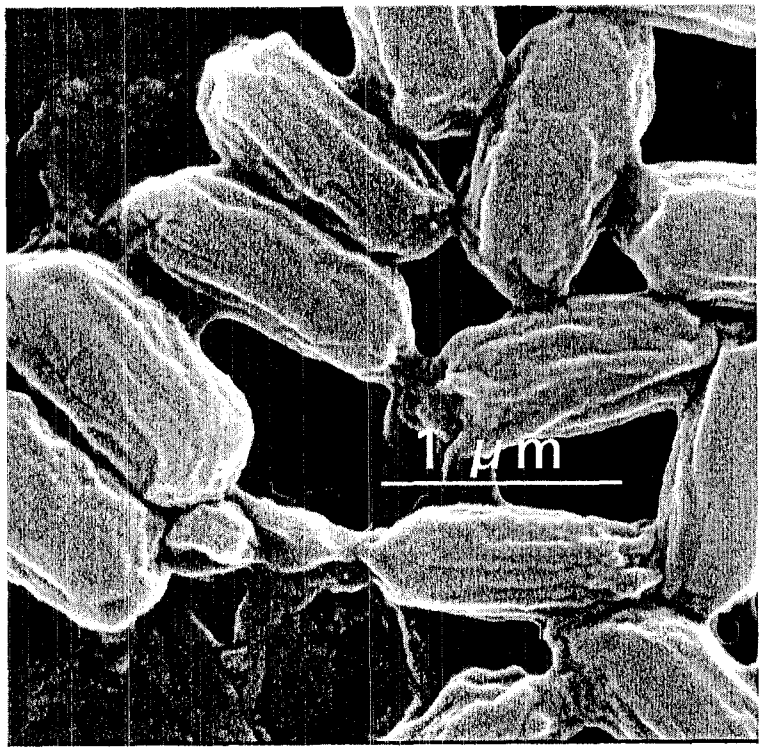


Figure 1. SEM of dried *Bg* spores on glass coupon. An aqueous solution of spores was placed on the SEM sample holder and allowed to air dry. Note that our preparation produces clumps of spores.

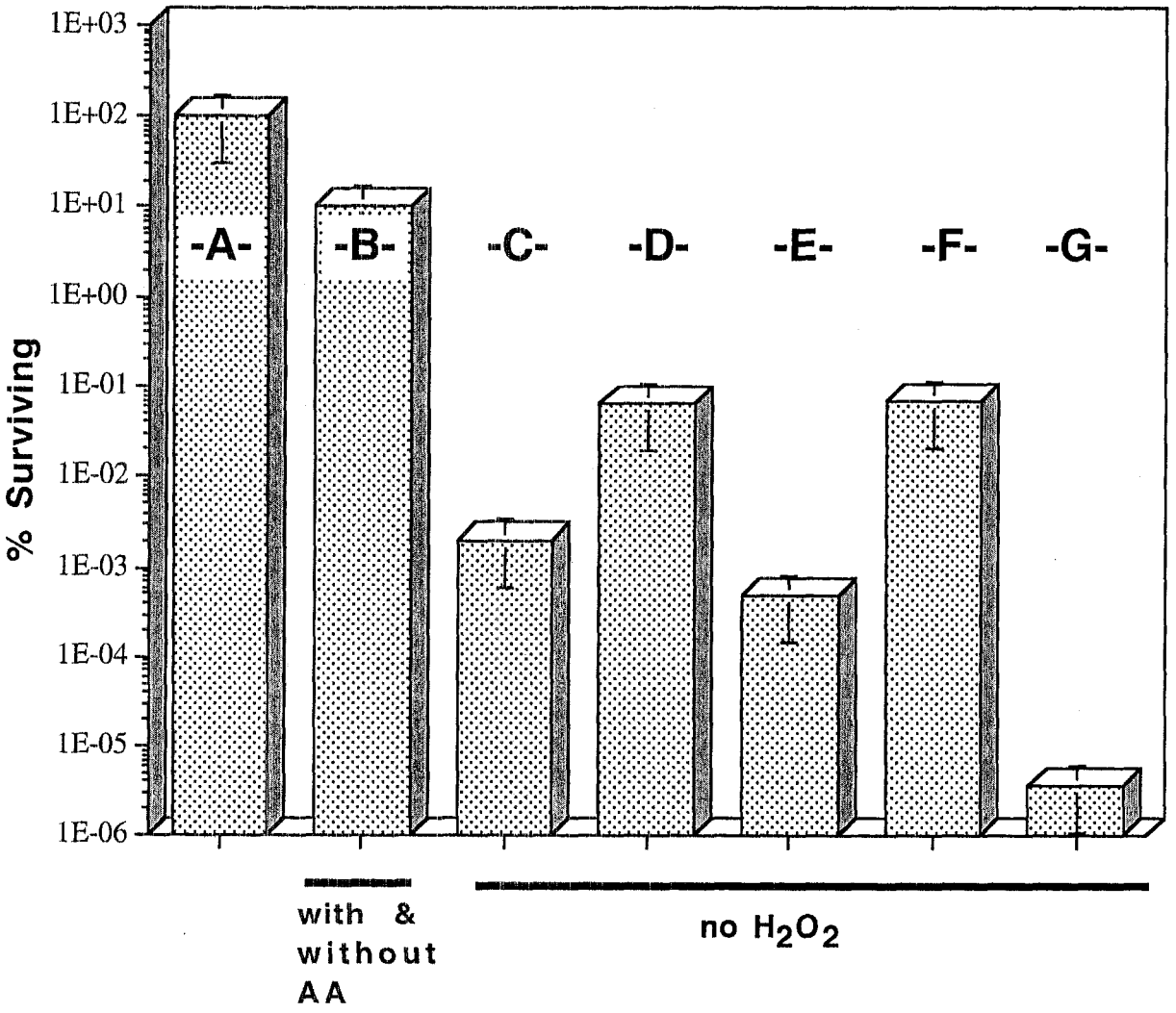


Figure 2. Comparison of various types of Fenton formulations on the disinfection (30-minute exposure time) of Bg spores suspended in solution. The percent surviving spores is shown for A) control, B) 0.6M Cu^{++}/0.1M H_2O_2 (with and without AA), C) 0.6M Cu^{++}/0.1M AA (no H_2O_2), D), 0.06M Cu^{++}/0.1M AA (no H_2O_2), E) 0.06M Cu^{++}/0.1M AA/2M NaCl (no H_2O_2), F) 0.06M Cu^{++}/0.1M AA/0.2M NaCl (no H_2O_2), G) 0.06M Cu^{++}/0.1M AA/2M NaCl/1% by weight surfactant (no H_2O_2). Note that conventional Fenton reagent using H_2O_2 (B) produces very little kill of *Bg* spores.

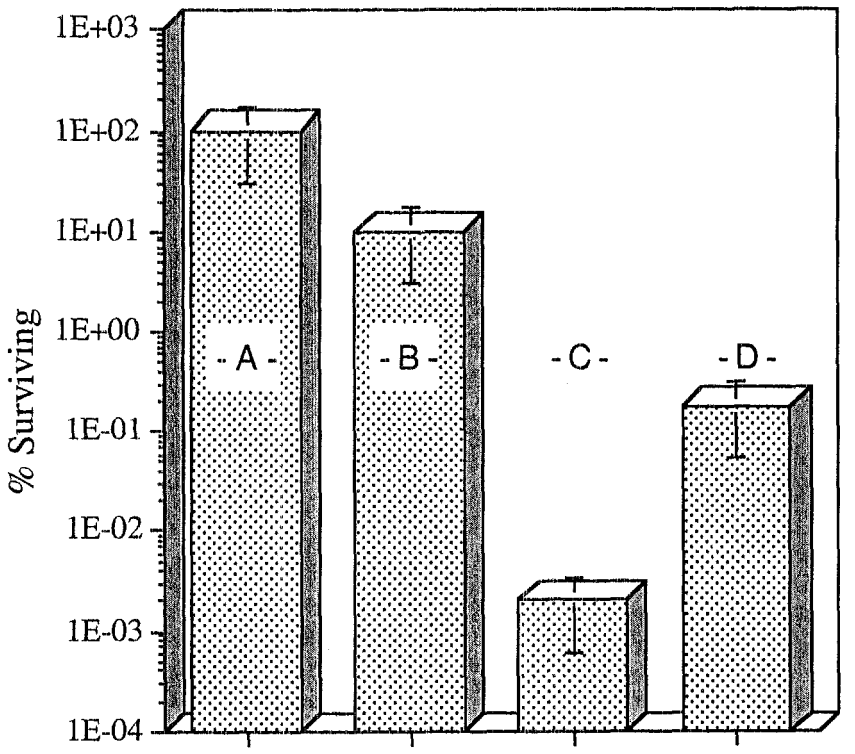


Figure 3. Comparisons of aerobic and anaerobic conditions kill effectiveness on *Bg* spores suspended in solution using anaerobic and aerobic solutions. A) control, B) 0.6M Cu^{++} + 0.1M hydrogen peroxide (no AA), C) aerobic 0.6M Cu^{++} + 0.1M AA, D) anaerobic 0.6M Cu^{++} + 0.1M AA. This set of data was obtained without the use of salt or surfactant.

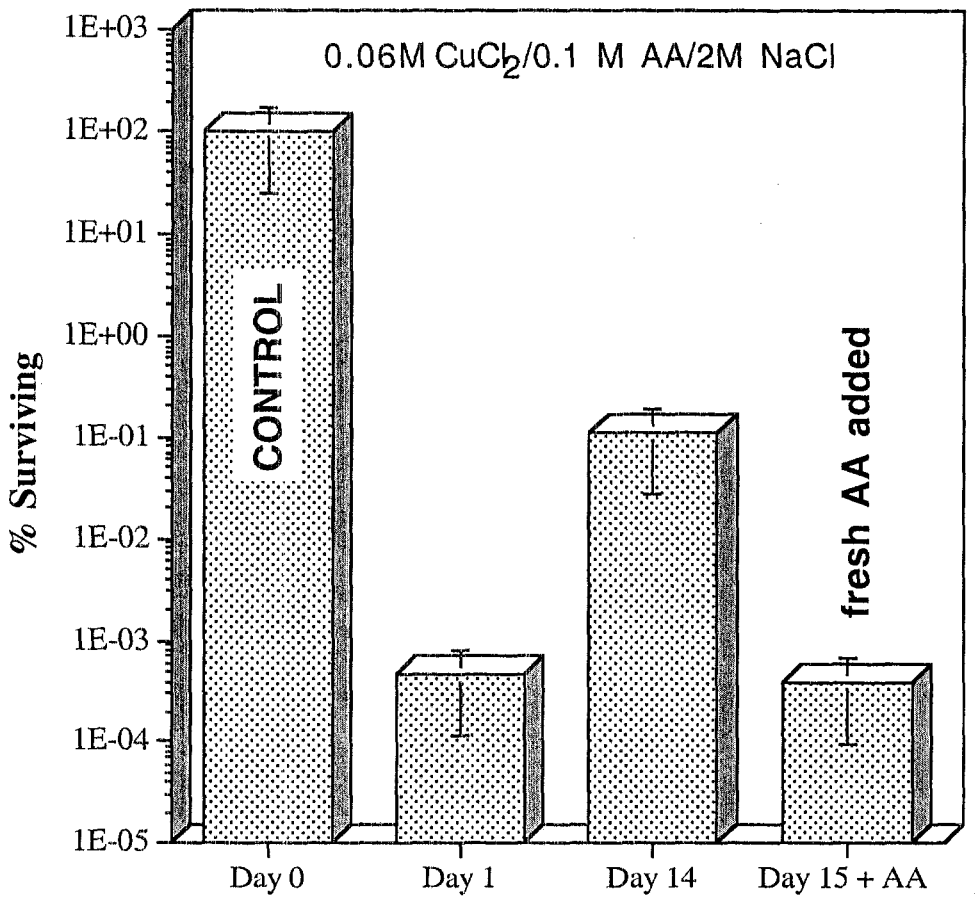


Figure 4. Aging study of aerobic modified Fenton reagent on Bg spores suspended in solution: Note that after 14 days the kill effectiveness has decreased by 2-3 log but that adding ascorbic acid on day 15 restores the kill effectiveness.

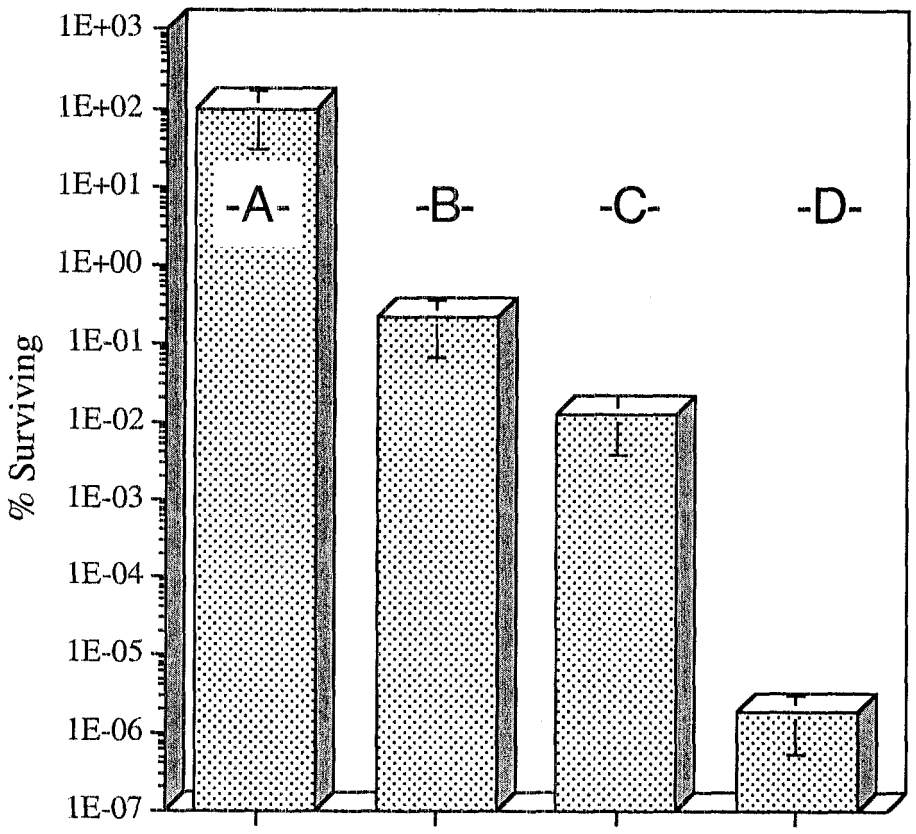
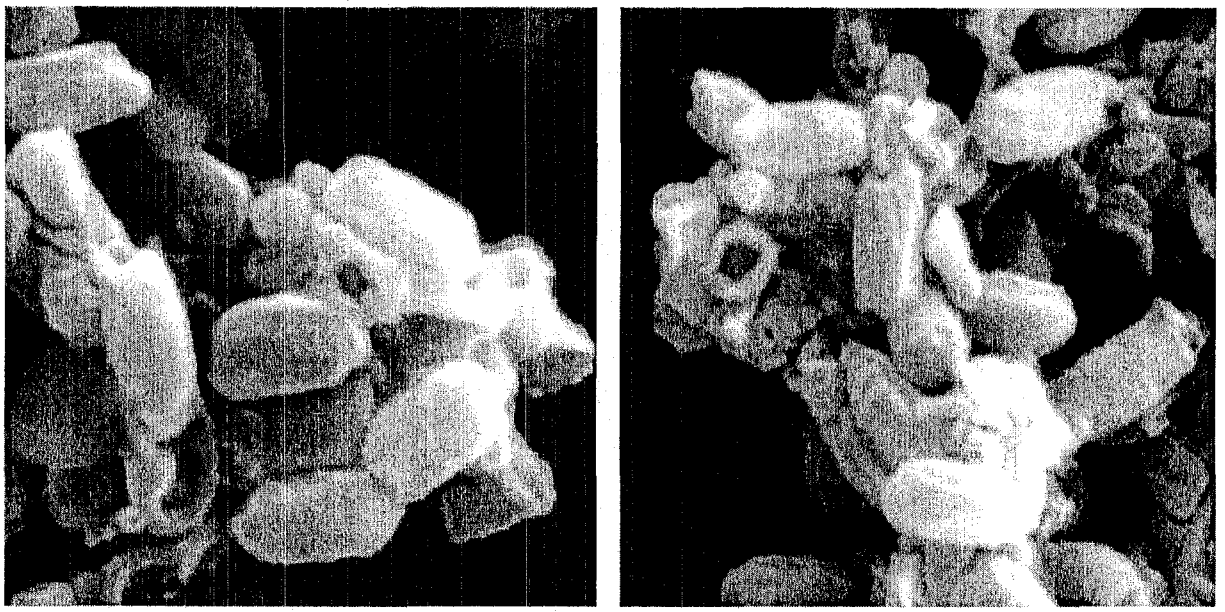


Figure 5. Copper oxidation state comparison for kill of *Bg* spores suspended in solution: A) control, B) Cu^{+}/NaCl/surfactant (no AA), C) Cu^{+}/NaCl/AA/NaCl/surfactant, D) Cu^{++}/NaCl/AA/NaCl/surfactant.



A B

Figure 6. SEM micrographs showing exposure time results of solution suspended *Bg* spores treated with modified Fenton reagent: 0.06M $CuCl_2$/ 0.1M ascorbic acid/2M NaCl/1% surfactant mixture for treatment times of 0.5 hours (A) and 16 hours (B).

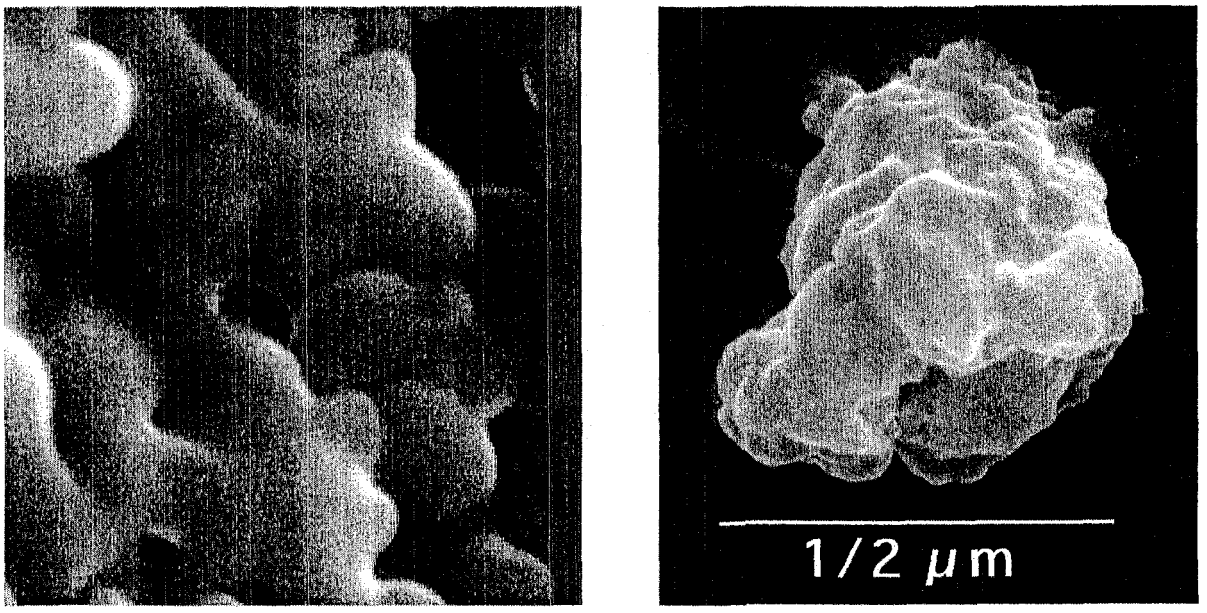


Figure 7. Left SEM shows the results of exposing spores mounted on a glass slide to 9000 ppm of ozone at 70% relative humidity. Right SEM shows spore SEM after 1-hour exposure to 1-% ozone in water at 25°C. Spore coat appears to be attacked where as the coat is not appreciably affected by the modified Fenton reagent (Fig. 6).

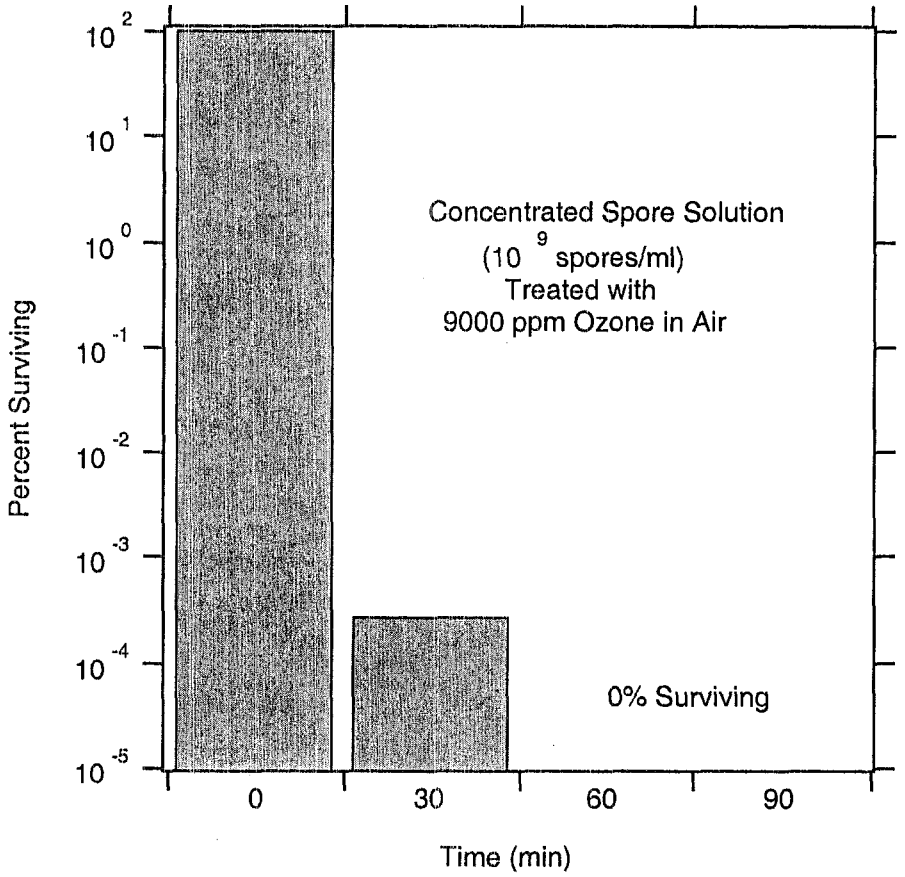


Figure 8. Results from ozonated water treatment of solution suspended *Bg* spores as a function of exposure time: ozonated air containing 9000 ppm O_3 was bubbled through the aqueous solution.

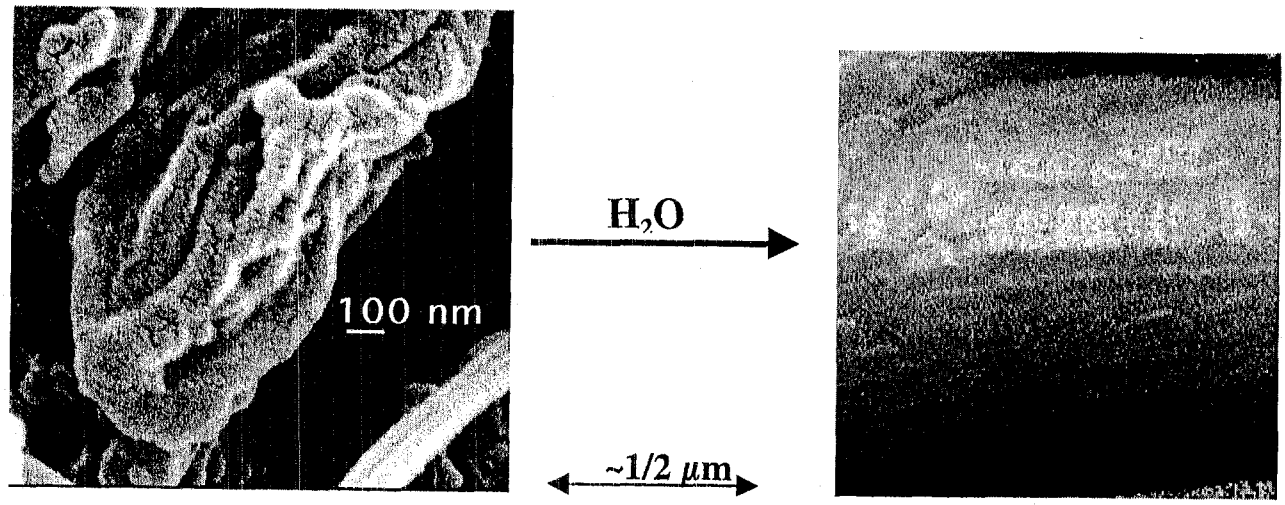


Figure 9. Scanning electron micrograph (SEM) of dry *Bg* spore being converted to hydrated spore as shown in the atomic force micrograph (AFM) taken with spore immersed in deionized water (ref 28). Spore was immersed in deionized water for several days before AFM was performed so no rate data was obtained on water uptake. Note swelling that occurred with water uptake.